Effect of Pumpkin Seed Enriched Probiotic Yogurt on Inflammatory Biomarkers, Insulin Resistance, and Hormonal Indices in Women with Polycystic Ovary Syndrome

SHORT SYNOPSIS

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by

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Certificate

I hereby declare that the Short Synopsis titled "**Effect of pumpkin seed enriched probiotic yogurt on inflammatory biomarkers, insulin resistance and hormonal indices in women with polycystic ovary syndrome**" being submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the Department of Nutrition & Dietetics, under the School of Allied Health Sciences of Manav Rachna International Institute of Research & Studies, Faridabad during the academic year 2024-2025. It is a bona fide record of original work carried out under the supervision of Dr. Madhvi Awasthi and has not been submitted in full or part to any other university or institute for a degree or diploma. Further, it is certified that the short synopsis is plagiarism-free and proof has been submitted.

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ABSTRACT

Polycystic Ovary Syndrome (PCOS) is a multifaceted endocrinological disorder affecting women of reproductive age, it is marked with high androgen levels, hormonal imbalances as well as metabolic disturbances. This study explores the intricate connections between gut dysbiosis, inflammation, insulin resistance, and PCOS development. The "Dysbiosis of the Gut Microbiota" (DOGMA) theory suggests that alterations in the gut composition may contribute to systemic inflammation and hyperinsulinemia which impacts androgen levels and impaired the development of follicles leading to classic symptoms of PCOS like menstruation irregularities, hirsutism, polycystic ovary morphology, and even infertility.Thus altering the gut environment with the help of probiotics and polyphenols can improve gut dysbiosis, leading to the lowering of low-grade inflammation, insulin signaling pathways, and androgen levels. This research investigates the impact of pumpkin seed enriched yogurt with BB-12 and La-5 bacterial strains, on inflammatory biomarkers, insulin resistance, and hormonal indices in young women with PCOS. The study employs a comprehensive methodology, including dietary assessments, anthropometric measurements, physical activity evaluations, and blood sample analyses. Four groups of participants will receive different interventions ranging from nutrition counseling to combinations of probiotic yogurt and pumpkin seeds. The research aims to elucidate the potential benefits of this nutritional intervention in managing PCOS by addressing gut dysbiosis and modulating inflammatory and hormonal pathways. Through data analysis using SPSS, the study seeks to contribute valuable insights into therapy methods for PCOS, emphasizing the significance of dietary interventions in improving the health outcomes of affected individuals.

Keywords: Gut dysbiosis, Hyperandrogenism, Inflammation, Insulin Resistance, Pumpkin seeds, Probiotic Yogurt.

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1. Description of Broad Area

Polycystic Ovary Syndrome (PCOS) is a complicated condition of the endocrine system that affects women of reproductive age. Although its etiology is unknown, theories suggest that the prognosis of PCOS is significantly influenced by environmental and genetic factors. It is a complex disorder, the effect of which goes beyond the gynecological system affecting the host's metabolism, immunity, insulin resistance, and type 2 diabetes. It is also correlated with dyslipidemia and cardiovascular diseases (Svendsen et al., 2012). It is marked by menstruation irregularities, hyperandrogenism, the presence of follicles in the ovaries, and infertility in most cases.

Diagnostic Criterion for Polycystic Ovary Syndrome (PCOS)

The diagnostic criteria used for PCOS are controversial with more than 2 criteria being used. National Institute of Health in 1990 suggested criteria for assessing polycystic ovary syndrome (Franks, 2006) revised in 2003 in Rotterdam known as the Rotterdam Criterion. The Rotterdam Criteria has been changed again, and according to the current criteria in 2023, the presence of two out of three conditions can qualify an individual as having polycystic ovarian syndrome: 1. Clinical hyperandrogenism (a modified Ferriman-Gallwey score ≥ 4 to ≥ 8) or biochemical hyperandrogenism (increased free or total testosterone) 2. indications of oligo-anovulation (cycles more than 35 days apart or 8 menstrual periods per year) 3. polycystic ovary morphology (more than 20 follicles per ovary or more than 10 cm^3 ovarian volume) (Christ & Cedars, 2023).

Adolescents face irregularities during menarche and also the transvaginal ultrasound is not possible to give a clear picture of the presence of follicles in the ovaries. The Pediatric Endocrine Society recommends different guidelines for the diagnosis of PCOS in adults and adolescents. Rotterdam and National Institute of Health (NIH) criteria are widely used to screen Polycystic Ovary individuals (Khan, 2007).

Pathophysiology and its Connection with Gut Microbiota

PCOS is a heterogeneous ailment involving multiple organs. There is a presence of high androgen levels and dysfunctions of ovarian functions. The exact pathophysiology of polycystic ovary syndrome is unknown. Still, in recent years research data correlated that changes in the gut composition (gut dysbiosis) can have an impact in the prognosis of PCOS through decreased permeability of the gut and leakage of lipopolysaccharide

(LPS) - a bacterial endotoxin into the systemic circulation which leads to systemic Inflammation leading to hyperinsulinemia and hyperandrogenism (Fig 1). The notion was originally introduced by Tremellen and Pearce (2012) and named it “DOGMA” (Dysbiosis of the Gut Microbiota). A study conducted in 73 patients found women with PCOS to have a less diverse microbiome as compared to normal women and less diversity was found to be negatively correlated with hyperandrogenism- the clinical feature of PCOS (Torres et al., 2018).

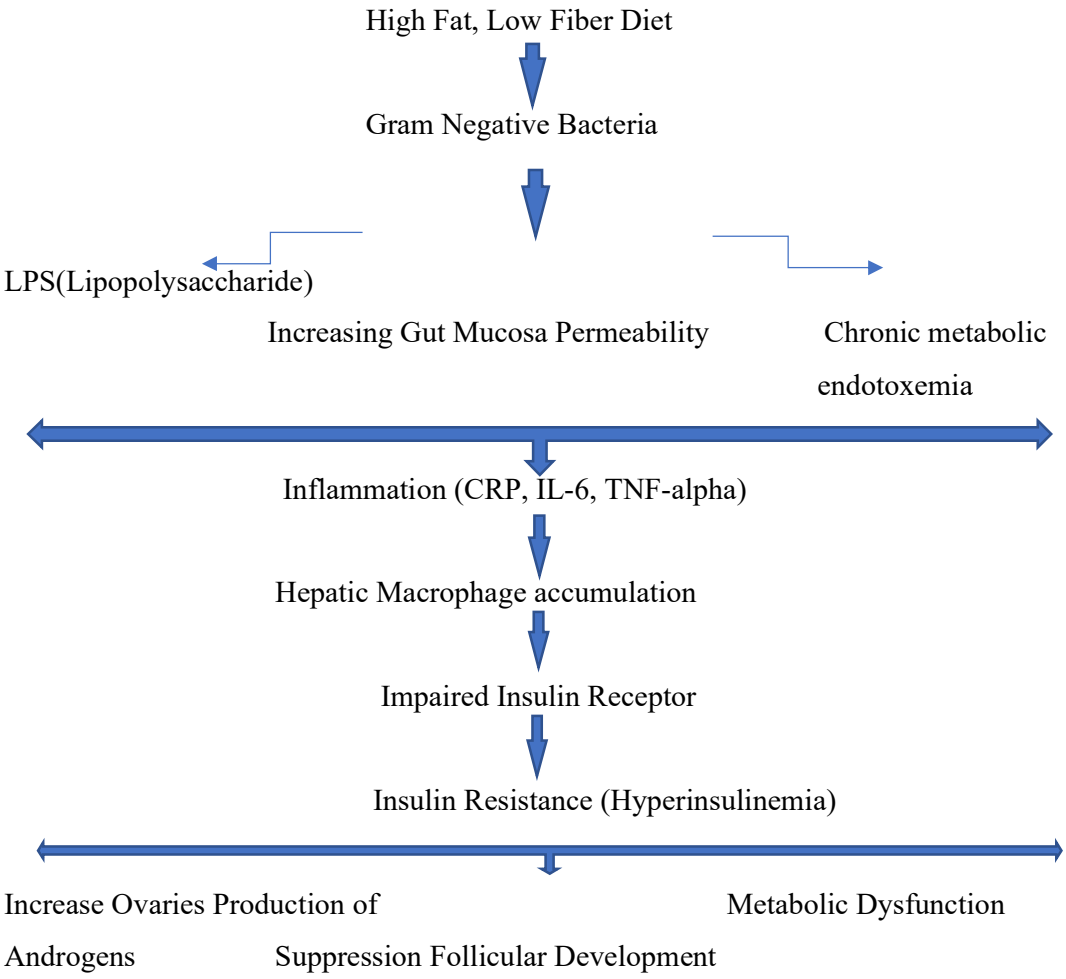

Fig 1: Leaky gut and its role in PCOS development (Ghanei et al., 2018)

According to DOGMA theory, the microbiome of the gut is composed of millions of bacteria, fungi, and protozoa along with their genes and metabolites, which maintain gut integrity. The disturbances in the gut environment lead to endotoxemia which results in inflammation and through hyperinsulinemia and multiple other pathways, affects the production of androgens leading to the development of PCOS. The main

pathways through which the gut microbiota impacts the progression of PCOS are inflammation, insulin resistance, and regulation of sex hormones (Fig 2).

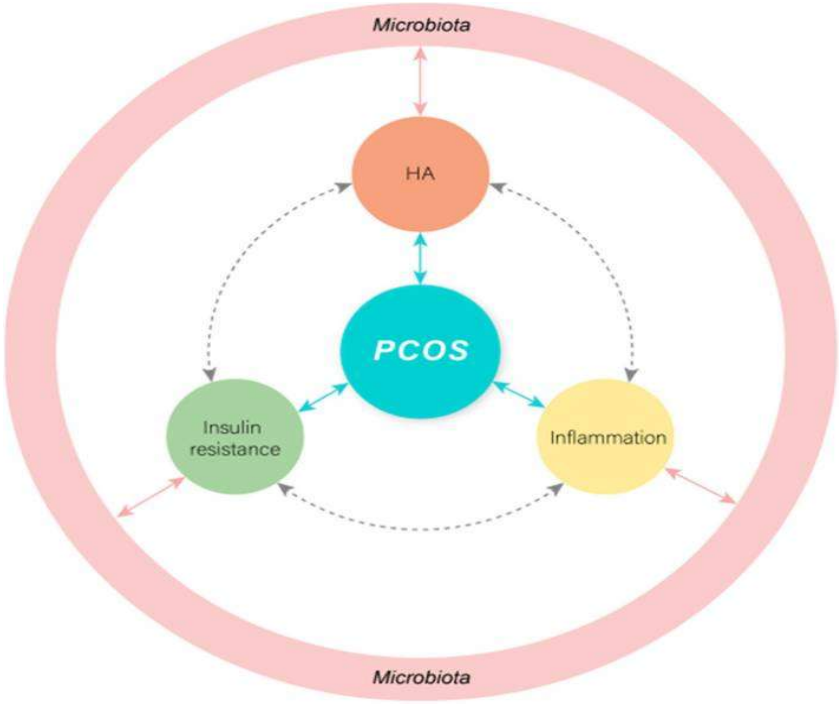

Figure 2: Inter-relationship between the Gut Microbiome, inflammation, insulin resistance, and hyperandrogenism (He & Li, 2020).

Diet has a major role to play in the development of intestinal dysbiosis. A diet high in fat and low in fiber over a long course of time increases the population of gram-negative bacteria and increases the permeability of the tight junctions. This leads to the outflow of lipopolysaccharide-based bacterial endotoxin into the systemic circulation. Low-grade chronic inflammation may affect the functioning of insulin receptors at various sites and lead to hyperinsulinemia.

Inflammation and hyperinsulinemia affect the production of androgens and impair the follicular development leading to hyperandrogenism and polycystic ovary syndrome. Together, these factors compound and are responsible for metabolic dysfunctions associated with PCOS.

2. Literature Review

Prevalence of Polycystic Ovary Syndrome (PCOS)

Across the globe, prevalence rates of polycystic ovary syndrome (PCOS) differ according to the criteria used among all Rotterdam criteria is the most acceptable one. As per the research data, the PCOS prevalence was found to be 1 in 10 women across the globe. Polycystic ovary syndrome affects about 4 – 20% of women in the reproductive age group (Deswal et al., 2020). Research done in China indicated that out of 915 women between the age group 18 and 45 years, 2.2% had polycystic ovarian syndrome (Chen et al., 2008). Another Community-based study in Sri Lanka showed a prevalence rate of 6.3% in women between the age group 15-39 years using RC criteria (Kumarappeli et al., 2008). According to Ganie et al. (2019) polycystic ovary syndrome in India has a prevalence rate of 3.7%-22.5% (according to different diagnostic criteria used and the population studied). The prevalence reported in 2014 in Rohtak, Haryana in North India was 6.8% in women between the ages of 16 and 45 years using Rotterdam criteria (Deswal et al., 2014). Another study conducted in Lucknow in North India on 1520 girls in the age group 18-25 years found that 3.7% were suffering from PCOS (Gill et al., 2012). With such a high prevalence rate which poses a risk to women's health, polycystic ovary syndrome imposes a social and economic health burden and should be recognized as an important health problem.

Gut Microbiome and Estrogen

Our gut is home to millions of bacteria, fungi, and viruses, all of these along with their metabolites and DNA are called the gut microbiome. According to Baker et al., (2017) a certain group of bacteria along with their genes are called "Estrobolome". Estrobolome has an important role in the metabolism of estrogen. Estrobolome produces an enzyme β-glucuronidase which de-conjugates the bound form of estrogen, leading to free estrogen, which is reabsorbed and re-circulated in the body. Thereby directly affecting the circulating levels of estrogen in the body. Excess estrogen or even low levels of estrogen can directly impact the alpha diversity of the gut and also influence the β-glucuronidase activity. Excess circulating levels of estrogen can lead to hyperinsulinemia and the progression of PCOS and endometriosis, thus gut microbiome and estrobolome may directly have an impact on the progression of PCOS (Qi et al., 2019).

Short Chain Fatty Acids and Hypothalamus-Pituitary-Gonad Axis (HPG)

When the gut microbes feed on the undigestible polysaccharide they produce Short Chain Fatty Acids (SCFA). There are many types of SCFA being produced like propionate, butyrate, and acetate. Short-chain fatty acids are essential in maintaining gut integrity and permeability and other activities such as metabolic management, hunger regulation, and immunological function. Among the SCFA produced in the gut lumen, butyrate acts as a substrate for the colonocytes. Colonocytes have an important role in the production of mucin, and butyrate is responsible for the regulation of Tight Junction Proteins (TJP) like claudin-1 and zonula occludens-1 (ZO-1) which maintain gut integrity and promote increased gut barrier functions. The improved gut barrier prevents the leaking of Lipopolysaccharide (bacterial endotoxin) into the systemic circulation thus preventing low-grade chronic inflammation and related insulin resistance.

SCFA binds to the L cells of the intestines to produce hormones such as GLP-1 and peptide YY (PYY). An Important and emerging role of GLP-1 is reported in the Hypothalamus-Pituitary-Gonad axis (HPG) (Fig 3).

These GLP-1 hormones stimulate the hypothalamus to produce Gonadotropin-releasing hormone (GnRH), which is responsible for the production of Luteinizing hormone (LH) by the pituitary gland. Luteinizing Hormone acts on the gonads to synthesize testosterone, which is aromatized to estrogen. The Hypothalamus-Pituitary-Gonad axis regulates follicular development and menstruation. A balanced gut microbiome may help in the synthesis of sufficient GLP-1 which has been correlated to have a positive effect on improving polycystic ovarian morphology through the gut-brain axis. (Giampaolino et al., 2021; Jensterle et al., 2019; Zhang et al., 2019).

Leaky Gut, Inflammation and Insulin Resistance

In 2012, Tremellen & Pearce hypothesized that dysbiosis of the gut microflora (DOGMA) can alter the permeability of the gut lining and could lead to leakage of lipopolysaccharide (LPS) (a bacterial endotoxin) into the systemic circulation. This leads to increased systemic inflammation and a heightened immunological response. This can cause the development of low-grade inflammation and insulin resistance. Therefore, dysbiosis in the gut microbiome and polycystic ovary syndrome can be correlated. The key mechanisms via which gut dysbiosis is thought to be implicated in

the pathophysiology of PCOS are chronic inflammation, insulin resistance, hyperinsulinemia, obesity, and oxidative damage.

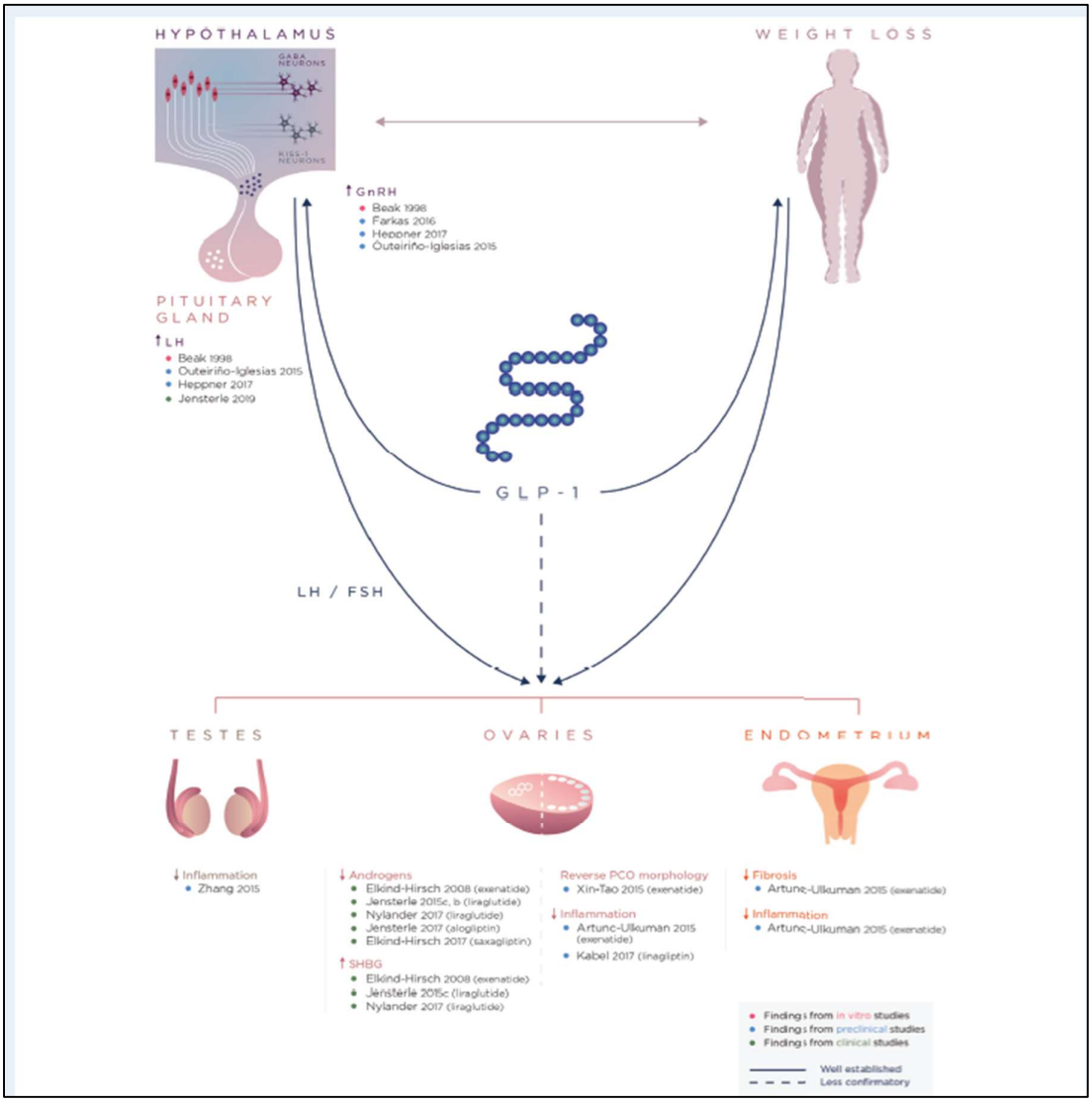

Fig 3: Role of GLP-1 in improving polycystic morphology (Jensterle et al., 2019)

The number of different species present in a single gut microbiota is called alpha diversity. A rich alpha diversity is linked to better health. Alterations in the gut microbiome have been identified and researched in PCOS patients. PCOS women were found to have a lower alpha diversity of the gut microbiota. The alpha diversity was found to be negatively correlated with the development of hirsutism, total testosterone, and hyperandrogenism which are classic symptoms of PCOS (Torres et al., 2018).

Numerous studies indicate that polycystic ovary syndrome patients have altered composition of the gut microbiome with a lower abundance of species *Bacteroidetes*

and *Firmicutes*, these bacterial species are linked with the production of SCFA (short chain fatty acids) and gut barrier integrity (Thackrey, 2019).

Long-term consumption of high sugar, high fat, and low fiber diet is associated with alteration in the gut microbiome composition increasing the number of gram-negative bacteria which leads to increased formation of Lipopolysaccharide (LPS) and reduced production of short-chain fatty acids (SCFA) leading to decreased permeability of the gut membrane due to decrease in mucin production by the colonocytes, which leads to the development of leaky gut syndrome (Tremellen & Pearce, 2012). Decreased permeability in the gut leads to leakage of LPS which binds to the Toll-Like Receptor 4 (TLR 4) cells present throughout the gastrointestinal cell. Activation of TLR-4 cells leads to the production of proinflammatory cytokines like TNF-alpha, IL-6, and CRP (He & Li, 2020).

Proinflammatory cytokines like TNF-alpha and IL-6 interfere with the insulin signaling pathways involving c Jun NH (2) terminal Kinase (JNK), IKK (enzyme involved in intracellular signaling), and Nuclear Factor kappa B(NF-kB) pathways which promote phosphorylation of serine kinase on Insulin Receptor Substrate-1(IRS-1) which interferes with the transport of glucose inside the cell and thus leading to insulin resistance (Moller, 2000; Hersoug et al., 2016).

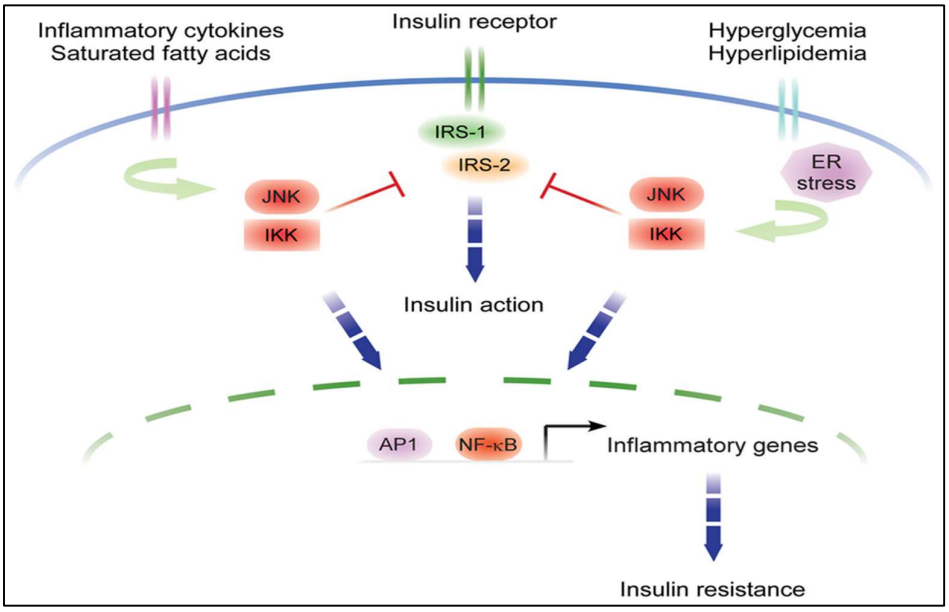

Fig. 4: Mechanism of inflammation and insulin resistance (Odegaard and Chawla, 2011)

Insulin Resistance, Inflammation, and Hyperandrogenism- Cascading Effect in PCOS

More than 50-90% of the patients with polycystic ovaries have insulin resistance (Venkatesan et al., 2001). Insulin resistance is the inability of insulin to regulate glucose metabolism efficiently, a higher amount of insulin would be released by the pancreatic cells to do the same amount of function leading to compensatory hyperinsulinemia (Franks, 2006). Hyperinsulinemia and insulin resistance work by different mechanisms to increase the production of androgens in excess. Insulin enhances the stimulating effects of luteinizing hormone on the theca cells of the ovaries to produce more androgens (Baillargeon & Carpentier, 2007). Hyperandrogenism leads to symptoms like hirsutism, acne, and androgenetic hair loss, excess testosterone in the ovaries also interferes with the follicular development leading to ovulatory dysfunction. It is correlated in many studies that women with PCOS are more susceptible to the androgen-increasing effects of insulin than normal women, and hence insulin resistance and hyperinsulinemia have a greater impact on the ovaries to produce more androgens and compromise ovarian function.

Another way excess insulin (hyperinsulinemia) and IR (insulin resistance) might affect androgen production is by inhibiting the release of SHBG (sex hormone-binding globulin) in the liver. SHBG is a main testosterone-binding protein and when SHBG release is reduced due to hyperinsulinemia, testosterone remains unbound or free, which can increase the hyperandrogenic activity leading to classical symptoms of PCOS like amenorrhea and hirsutism.

Therefore, the treatment of polycystic ovary syndrome can involve addressing the gut dysbiosis with the intervention of probiotics and anti-inflammatory foods which would improve the underlying inflammation and insulin resistance leading to reversal of the symptoms.

Probiotics, Inflammation and Insulin Resistance

"A yogurt is defined as the fermented milk containing symbiotic cultures of *Streptococcus thermophilus* subsp. *salivarius* and *Lactobacillus delruekii* subsp. *bulgaricus*" as defined by Codex Alimentarius International Food Standards

According to World Health Organization (WHO) defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host."

“Probiotic yogurt is contain multiple bacteria, including *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Lactobacillus casei*, and/or *Lactobacillus rhamnosus* among others. These bacteria, which are suggested to be beneficial for human health, are present in probiotic yogurt in addition to *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, which are the most frequent microorganisms used to produce conventional yogurt” (Barengolts et al., 2019).

Yogurt is a popular fermented food that is globally accepted. It is a great vector of probiotic administration. It contains proteins and the all-essential amino acids and a positive correlation has been observed in human studies between yogurt protein and satiety and weight loss. Yogurt is also a rich source of important minerals like calcium which itself has many beneficial effects in women and magnesium and phosphorous which have been associated with improving insulin sensitivity (Veldhorst et al., 2008).

Bacterial strains in the yogurt may have different survival efficiency in gastrointestinal transit. S*treptococcus thermophilus* and *lactobacillus bulgaricus*, (present in conventional yogurt) reach a low fecal concentration of 10^4 to 10^6 CFU/g feces, strains like *Bifidobacterium animalis* survive the gastrointestinal transit and reach the colon with an abundance of 10^8 CFU/g feces (Veiga et al., 2014).

The mechanism by which probiotics work is by improving the abundance of the microbes important for SCFA production thereby reducing the pro-inflammatory cytokines and improving intestinal permeability. SCFA also plays a role in reducing inflammation by improving intestinal permeability by regulating zonulin occludin-1 and claudin -1 (proteins present at the tight junction) and increasing the mucin production by colonocytes, thereby reducing the circulating endotoxins, leading to lowering inflammation (Kim et al., 2018).

Short-chain fatty acids like propionate, acetate, and butyrate are required in the production of Glucagon Peptide-1 (GLP-1) hormone in the L cells of the intestines which are known to regulate appetite and also improve the polycystic ovary morphology through the Gut-Brain axis.

Tremellen & Pearce in 2012 postulated a link between chronic inflammation, and insulin resistance with the development of PCOS. Alteration of the gut microbiome is responsible for increased intestinal permeability and increased growth of gram-negative bacteria which leads to escape of Lipopolysaccharide (a bacterial endotoxin) into the circulation activating the immune response and leading to chronic inflammation and

through various biochemical pathways leading to hyperinsulinemia and hyperandrogenism. Improving gut dysbiosis can improve the symptoms of PCOS.

A study on 64 type 2 diabetic individuals was conducted to study the effect of probiotic yogurt containing *Bifidobacterium lactis* Bb12 and *Lactobacillus acidophilus* La5 or conventional yogurt (curd) on cholesterol profile for 6 weeks. The subjects were randomly assigned to 2 groups. Group I received 300 gm of probiotic yogurt and the other (group II i.e. control) received 300 gm of conventional yogurt (curd) for 6 weeks. Probiotic yogurt reduced total cholesterol by 4.54% and 7.45% decrease in LDL-C compared to the control group (Ejtahed et al., 2011).

Another study was conducted on 44 type 2 diabetic patients. The patients were divided into two groups and were given 300 gm of conventional yogurt (curd) and 300 gm of probiotic yogurt for eight weeks. To investigate the effect of yogurt consumption on inflammatory markers such as TNF-alpha, CRP, and IL-6 levels. The study found that probiotic yogurt significantly reduced HbA1c levels ($p= 0.032$) and TNF-alpha levels ($p= 0.040$) (Mohamadshahi et al., 2014).

According to a metabolomics analysis, a study found the *Bifidobacterium animalis* subsp *lactis* to be associated with a production of 13 specific metabolites, which also included 3-hydroxy octanoic acid which is associated with lowering inflammation (Le Roy et al., 2022).

Pumpkin seeds, Phytoestrogens, and Insulin Resistance

Pumpkin seeds have gained popularity due to their nutraceutical properties and functional nutrients. Pumpkin seeds possess estrogenic-like properties, owing to its lignans phytoestrogen content- secoisolariciresinol (SECO) and lariciresinol, it has hypoglycemic properties due to the presence of high levels of polyphenols– α-spinasterol and β-sitosterol and anti-inflammatory activity owing to the high content of Vit E mainly in the form of Gamma-tocopherol.

The phytoestrogen content of pumpkin seeds is 256 mg/100 g (Lestari & Meiyanto, 2018) of which SECO is 210µg/kg, Vit E content in the form of gamma-tocopherol is 620mg/kg dry weight (Murkovic et al., 1996) and polyphenols, β-sitosterol and α-spinasterol content in pumpkin seeds oil is 100mg/kg and 745 mg/kg respectively (Dotto & Chacha, 2020).

PCOS is characterized by excess production of testosterone in the ovaries leading to classic symptoms like irregular menstruation and hirsutism.

Lignans like secoisolariciresinol (SECO) and lariciresinol, present in pumpkin seeds are converted to enterolactone and enterodiol by the activity of bacteria in the gut involving a series of reactions. Enterolactone and enterodiol have a structure similar to estrogen and therefore can compete for estrogen receptor sites, when they bind to the estrogen site, they produce a weak estrogen response, and owing to this property they prove to be anti-estrogenic (Ervin et al., 2019).

Lignans extracted from pumpkin seeds have been shown to have a high affinity for Estrogen receptor-α (Richter et al., 2013). Another study conducted on ovariectomized Sprague drawley rats showed the effect of SECO and lariciresinol, two important lignans extracted from pumpkin seeds on estrogenic activity. A high docking ability of the lignans from pumpkin seeds to the estrogen receptor sites will help in exerting anti-estrogenic activity, thus improving the effect of excess estrogen as in PCOS (Lestari et al., 2019).

Lignans are also proposed to increase the production of SHBG levels in the liver which binds to excess testosterone. Lignans also inhibit the activity of 5α-reductase which is responsible for converting testosterone to dihydrotestosterone, thus improving the symptoms of androgen excess in PCOS (Mehraban et al., 2020). According to Majeed, et al. (2022) pumpkin seeds have a high content of alpha-spinasterol which is a potent anti-diabetic, the main mechanisms involved in the anti-diabetic is by the increase in the secretion of insulin, increased uptake of glucose in the muscle cells and reduction of insulin resistance.

Pumpkin seeds were also shown to strongly inhibit α-amylase and α-glucosidase activity, thus exhibiting anti-diabetic properties. Further, in the same study, a group of 60 Indian women with metabolic syndrome were supplemented with 5gms of pumpkin seeds for 60 days and, it was reported to show a significant reduction in fasting plasma glucose (Monica et al., 2022).

In another study, the acute consumption of 65 gm of pumpkin seeds (and not the extract), for 3 consecutive days was shown to markedly improve the post-prandial glycemic response, whereas the same amount of flaxseed did not show a significant difference. Post-prandial hyperglycemia is an accurate measure of metabolic dysfunction of diabetes and can affect the β-cells dysfunction, affecting the production of insulin (Candido et al., 2018).

Vit E is a potent antioxidant and is associated with reducing oxidative damage and inflammation. PCOS is an endocrinopathy marked by inflammation which leads to

increased oxidative stress (OS), and insulin resistance. Increased oxidative in PCOS individuals is associated with infertility.

Gamma-tocopherols have an emerging role in preventing inflammation because they trap the reactive nitrogen species by getting converted to 5-nitro-gamma- tocopherol whereas alpha-tocopherols cannot trap reactive nitrogen in this manner (Jiang et al., 2022).

Gamma-tocopherols alone and in combination were shown to improve oxidative stress markers and inflammation in subjects with metabolic syndrome (Devaraj et al., 2008).

Various intervention studies indicate a positive correlation between vitamin E consumption and improvement in hormonal functions. A recent meta-analysis and systemic review (Tefagh et al., 2022) has shown that vitamin E administration raises follicle-stimulating hormone and progesterone levels while decreasing insulin and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) levels, therefore Vit E supplementation can be used to improve the metabolic profiles, inflammatory markers and hormonal parameters in women with PCOS.

Probiotic yogurt, Pumpkin seeds, and Combined effect

Polyphenols present in various foods like seeds, grains, fruits, and vegetables have been shown to increase the growth of Lactobacillus bacteria, which is a beneficial bacteria and aids in the digestion and absorption of antioxidants. Thus, combining a functional food containing polyphenols and Lactobacillus bacteria can have beneficial effect on the microbiome which leads to positive impact on human health (Piekarska-Radzik et al., 2021).

In a recent study on the fermentation of pumpkin seed flour by *Lactobacillus acidophilus*, it was reported that the fermentation decreased the phytic acid content, increased the breakdown of phenolic compounds into small compounds, and also improved the mineral bio-availability, thus *Lactobacillus acidophilus* was shown to act on polyphenols present in pumpkin seeds (Kumari et al., 2021). Lignans in pumpkin seeds and *Lactobacillus acidophilus* in probiotic yogurt thus possess synergistic ability which can help improve the gut microflora abundance in the host.

An observational study conducted on 7169 Spanish participants from 2003 to 2009, reported that a high consumption of lignan and yogurt was correlated with lesser cardiovascular risk factors. The study indicated that participants consuming more than

0.67 mg/day of lignan had significantly lower glucose levels in plasma (Creus-Cauadro et al., 2017).

A recent study was conducted to evaluate the effect of flaxseed-enriched yogurt on glycemic status and cardiovascular risk factors in type 2 diabetic individuals. Fifty-seven diabetic individuals were enrolled and were divided into 2 groups, one receiving 200gm yogurt and the other receiving a combination of 200gm yogurt and 30 gm flaxseed for 8 weeks. After 8 weeks there was a significant reduction in HBA1c, total cholesterol, and triglyceride concentration in the flaxseed and yogurt combined group which is suggestive of lignans and probiotics showing a synergistic effect (Hasaniani, 2019).

Another study conducted on diabetic individuals evaluated the effect of consumption of Pumpkin and Probiotic yogurt alone and in combination on blood glucose, lipid profile, and inflammatory markers.

A total of 80 diabetic individuals were divided into 4 groups, one receiving 100 gm pumpkin, the second receiving 150 gm of probiotic yogurt, the third receiving 150 gm of probiotic yogurt, and 100 gm pumpkin, and the fourth as a control receiving dietary advice. The combined group and probiotic yogurt group showed a statically significant improvement in the cholesterol levels. All interventions led to a significant decrease in the HbA1c and CRP levels, thus explaining the potential role of pumpkin and probiotics in the management of inflammation and insulin resistance (Bayat et al., 2016)

A study conducted on forty-four PCOS women between the age group 18-45 years showed the impact of flaxseed supplementation with lifestyle modification in comparison to lifestyle modification alone on metabolic status. It was reported that 30gm flaxseed consumption along with lifestyle modification showed a significant reduction in HOMA-IR, triglycerides, hs-CRP, and an increase in QUICKI index and HDL levels, along with significant weight reduction, thereby suggesting that 30gm of seeds along with lifestyle modification can be an effective way to manage biochemical as well as anthropometric markers in PCOS women (Haidari et al., 2020).

A recent study conducted on Swiss mice showed the beneficial effect of a synbiotic combination of probiotic yogurt with yacon flour to have significant results in reducing body weight, improved insulin signaling, decreased fasting glucose, and inflammatory markers. The study supported the use of a combination of probiotic yogurt and yacon (prebiotic) to have beneficial effects (de Abreiu et al., 2022).

Another study investigated the effect of probiotic capsules (*Lactobacillus acidophilus*, *Lactobacillus casei,* and *Bifidobacterium bifidum* in the strength $2*10^9$ CFU/g) on hormonal profile, inflammation, and oxidative markers. It concluded that probiotic supplementation for 12 weeks significantly improves Sex Hormone Binding Globulin (SHBG) and plasma TAC (Total antioxidant capacity) as compared to the placebo. It also showed a significant decrease in serum total testosterone (hormone marker), CRP levels, and malondialdehyde the markers of oxidative stress, but the probiotic supplementation did not show any significant changes in metabolic profiles (Karameli et al., 2018).

Another study conducted on 60 women with Polycystic ovary syndrome showed the beneficial effect of the combination of probiotic capsules and selenium. The capsules contained four strains of bacteria namely *Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus fermentum,* and *Bifidobacterium bifidum* ($2*10^8$), and selenium in the dosage of 200 mcg/day. The co-supplementation showed significant improvement in hormonal markers like total testosterone and hirsutism index as compared to the placebo, and significant improvement in inflammatory markers like c-reactive protein and malondialdehyde levels as compared to the placebo (Jamilian et al., 2018).

The Rationale for the quantity of pumpkin seeds

The study conducted with flaxseed and yogurt has also suggested an intake of 30 gm of seeds in a day to improve glycemic control including insulin resistance and hormonal indices in women with PCOS (Bayat et al., 2016).

A study on combined seeds in PCOS women indicated that a combination of 30 gm of flaxseeds and pumpkin seeds in the follicular phase and a combination of sesame seeds and sunflower seeds in the luteal phase of menstruation, showed significant changes in hormonal health and suggested consumption of 30 gm of seeds along with portion control diet as an effective strategy in the management of PCOS (Rasheed et al., 2023).

Another study conducted with thirty PCOS patients suggested that 15 gm of flaxseed consumed with milk for 3 months can have a beneficial effect on peripheral follicles in the ovary and also reduce ovarian volume (Farzana et al., 2015).

With the above considerations, it is suggested that seeds in a quantity between 15-30 gm in a day can produce beneficial effects. Indian Council of Medical Research and the National Institute of Nutrition, have provided the MyPlate concept to reduce the risk of

non-communicable diseases. It recommends a dietary allowance for nuts and seeds as 30 gm in a 2000 kcal diet (Hemalatha, 2023).

Therefore pumpkin seeds are readily available and attractive nutraceuticals that can be used in PCOS patients along with probiotic yogurt to exert a synergistic effect in the individual and are proposed to help in the management of underlying inflammation, insulin resistance, and improve hormonal balance.

3. Research Gaps

PCOS is a multifaceted endocrinological disorder impacting fertility, ovaries, and metabolic health. Gut dysbiosis has been associated with the disorder's etiology and progression, with metabolic endotoxemia resulting from long-term low-fiber and high-fat diets leading to systemic inflammation and insulin resistance. While studies focus on the use of probiotics to enhance gut health and reduce inflammation, the nutraceutical properties of pumpkin seeds in women with PCOS remain underexplored. Pumpkin seed-enriched probiotic yogurt will have the properties of probiotics in yogurt and phytochemicals in pumpkin seeds are a less-explored area in PCOS. This study aims to evaluate the effect of pumpkin seed-enriched probiotic yogurt on inflammatory biomarkers, insulin resistance, and hormonal indices in women with PCOS.

4. Problem Statement

With the increasing prevalence of PCOS among women, it increases the chances of co-morbidities like metabolic syndrome, diabetes, and infertility which raises the health burden of society. Probiotics through yogurt and polyphenols through pumpkin seeds when working synergistically can serve as an easy and cost-effective approach to improve the health status of women suffering from PCOS.

5. Research Objectives

1. To compare the effectiveness of probiotic yogurt vs pumpkin seeds vs pumpkin seed-enriched probiotic yogurt with a balanced diet on inflammatory biomarkers.
2. To compare the effectiveness of probiotic yogurt vs pumpkin seed vs pumpkin seed-enriched probiotic yogurt with a balanced diet on insulin resistance.
3. To compare the effectiveness of probiotic yogurt vs pumpkin seed vs pumpkin seed-enriched probiotic yogurt with a balanced diet on hormonal indices.
4. To assess the impact of probiotic yogurt vs pumpkin seeds vs pumpkin seed-enriched probiotic yogurt with a balanced diet on anthropometric measures and adherence to the intervention.

Aim of the Study

This study aims to assess the effect of pumpkin seed-enriched probiotic yogurt on inflammatory biomarkers, insulin resistance, and hormonal indices in women with Polycystic Ovary Syndrome.

Hypothesis

The following set of null and alternate hypotheses are proposed to address the objectives written above:

Null Hypothesis (Ho1): There will be no effect of pumpkin seed-enriched probiotic yogurt on inflammatory biomarkers, insulin resistance, and hormonal indices in women with Polycystic Ovary Syndrome.

Alternate Hypothesis (Ha1): A statistically significant difference will exist in inflammatory biomarkers, insulin resistance, and hormonal indices of selected PCOS patients after consumption of pumpkin seed-enriched probiotic yogurt.

6. Research Methodology

Study Design

The present study will be a randomized controlled clinical trial, the protocol has been approved by the institutional ethics committee (Ref no. ED and Dean Res./1284).
For the study written informed consent will be obtained from the participants.

Area of the Study:

The proposed study "Effect of pumpkin seed enriched probiotic yogurt on inflammation, insulin resistance, and hormonal indices in women with PCOS," will be conducted at gynecological clinics, dermatological clinics, and community settings in Central Ghaziabad and East Delhi.

Study Population: The study will focus on adult females in the age group of 20 to 29 years. The exclusion criteria and inclusion criteria for the study will be:

Inclusion Criterion:

- Adult females aged 20-29 years.
- Females screened for PCOS using the 2023 Rotterdam criteria, according to the criteria the presence of two out of three conditions can qualify an individual as having polycystic ovarian syndrome: 1. Clinical hyperandrogenism (a modified Ferriman-Gallwey score ≥ 4 to ≥ 8) or biochemical hyperandrogenism (increased free or total testosterone) 2. indications of oligo-anovulation (cycles more than 35 days apart or 8 menstrual periods per year) 3. polycystic ovary morphology (more than 20 follicles per ovary or more than 10 cm^3 ovarian volume) (Christ & Cedars, 2023)
- Females visiting gynecological or dermatological clinics or community settings.
- Females with PCOS exhibit moderate physical activity according to GPAQ.

Exclusion Criterion:

- Females with PCOS on any antibiotic treatment in the past 6 months.
- Females with PCOS who consume probiotic supplements.
- Females with PCOS on hormonal birth control pills.
- Females with any underlying co-morbidities like diabetes, CVD, and hypertension.
- Females with PCOS with a history of pregnancy and lactation in the past 6 months.

Sample Size:

The sample size, calculated using G Power, is 108 for 4 groups at a 95 percent confidence level (at 0.05 percent significance level, and 85 percent power with an effect size of 0.3). Considering a 15% dropout rate, the adjusted sample size is 124. There would be 31 in each group (A total of four groups). (Fig 5)

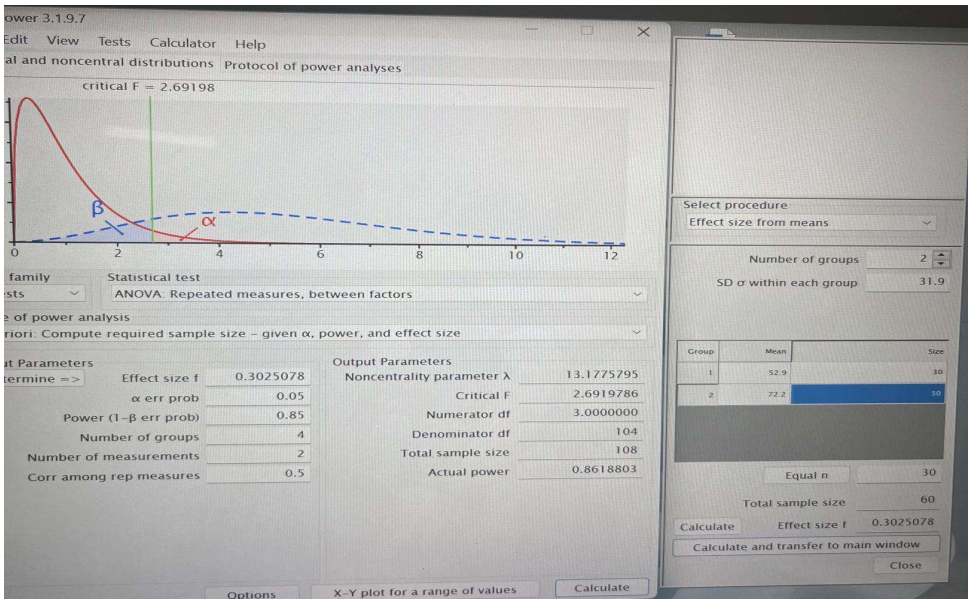

Fig 6: Sample size calculation using G-Power

Sampling Technique:

Random sampling will be employed, categorizing women with polycystic ovary syndrome visiting different clinics, followed by random participant selection and allocation to the control group and three intervention groups.

Screening of the PCOS patients

Physical activity levels will be measured using the Global Physical Activity Questionnaire (GPAQ) to screen females with PCOS as per the inclusion criteria of the study. Adult females in the age group 20-29 years with PCOS and moderate physical activity will be included in the present study (Armstrong & Bull, 2006).

Data Collection

Anthropometric Measurement

The screened adult females with PCOS will be studied for their anthropometric measurements, including body weight, height, Body Mass Index (BMI), hip and waist

circumference (Wang et al., 2012). This will be done at the beginning and after 12 weeks of the dietary intervention to assess the changes in body indices as the secondary outcome of the study.

Height will be measured using a rolling ruler wall-mounted stature meter to the nearest 0.5cm.

Body weight will be measured to the nearest 0.1kg using a portable weighing scale after adjusting to zero.

Body Mass Index (BMI) is calculated using the following formula

$$\text{Body Mass Index} = \frac{\text{Weight in kg}}{\text{Height in m}^2}$$

Waist circumference is the measure of abdominal obesity and will be measured by measuring the distance around the waist half an inch above the umbilicus measuring tape to the nearest 0.1 cm (Misra & Vikram, 2004).

The values will be recorded in triplicate to avoid the error.

Biochemical Assessment

Selected adult females with PCOS will be then studied for the following parameters at the beginning and after 12 weeks of the dietary intervention. For this, blood samples will be drawn in the follicular phase of the cycle on the 1st to 3rd day of their menstruation if not amenorrhoeic after 12 hours of fasting by laboratory professionals:

- Interleukin-6
- C-reactive Protein (CRP)
- Total Testosterone
- SHBG (Sex Hormone Binding Globulin)
- Free androgen Index (FAI)
- Fasting insulin
- Fasting Glucose
- HOMA-IR (using fasting glucose and fasting insulin)

Dietary assessment:

Dietary assessments of selected adult females with PCOS will be conducted using a 24-hour dietary recall method and a standardized Food Frequency Questionnaire (FFQ) at the beginning and after 12 weeks of the study (Telles et al., 2016). The inference will

be drawn at the end of the study to observe the changes in diet and the impact of nutritional counselling.

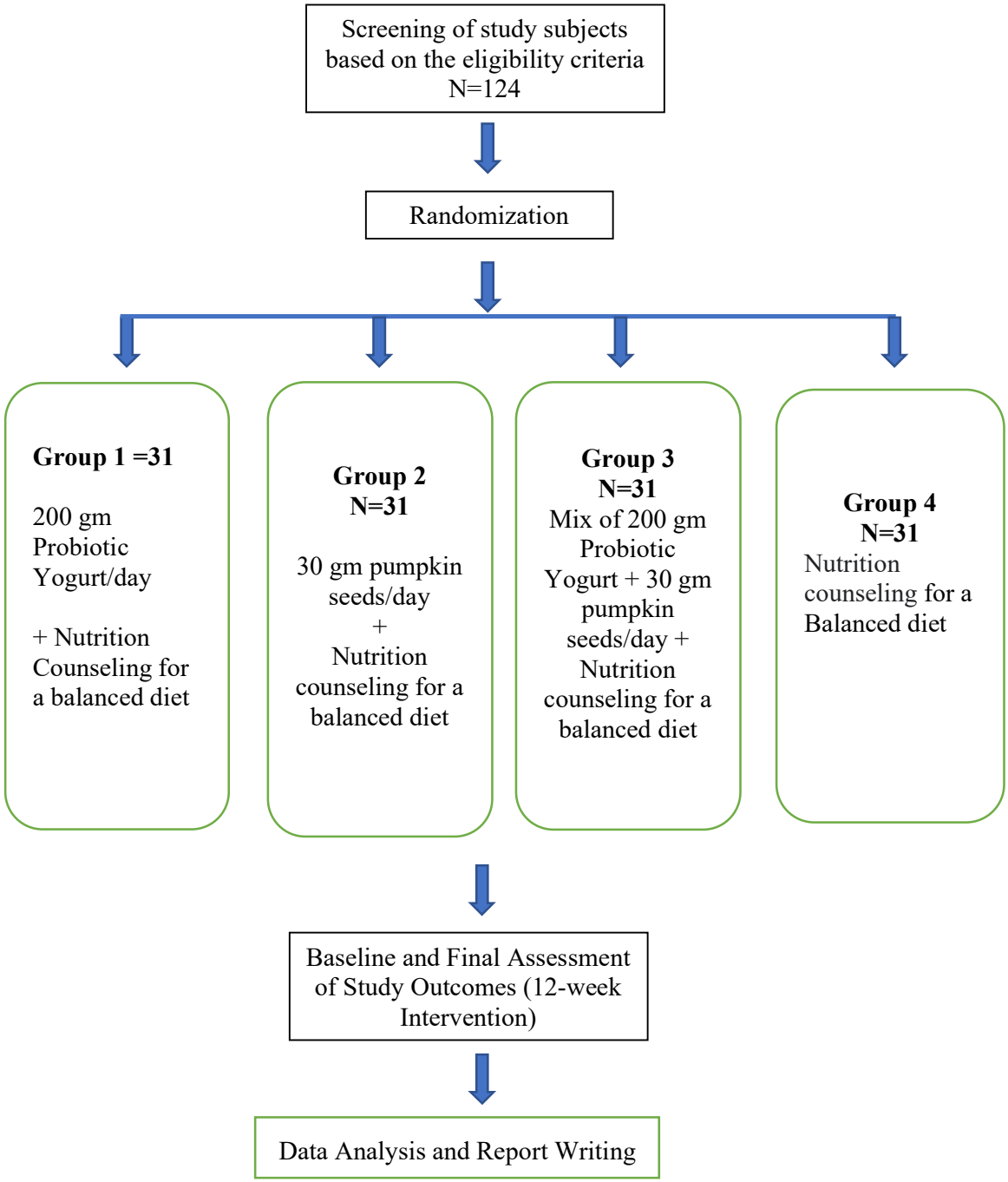
Screening of study subjects based on the eligibility criteria N=124
Randomization
Group 1 =31
200 gm Probiotic Yogurt/day
+ Nutrition Counseling for a balanced diet
Group 2 N=31
30 gm pumpkin seeds/day + Nutrition counseling for a balanced diet
Group 3 N=31
Mix of 200 gm Probiotic Yogurt + 30 gm pumpkin seeds/day + Nutrition counseling for a balanced diet
Group 4 N=31
Nutrition counseling for a Balanced diet
Baseline and Final Assessment of Study Outcomes (12-week Intervention)
Data Analysis and Report Writing

Fig 6: Study flowchart: Recruitment, group allocation, counseling, and dietary interventions illustrated.

Dietary Intervention:

For the current study, the intervention will include, dehulled pumpkin seeds and market-available yogurt. Pumpkin seeds will be purchased from the local market. Yogurt will also be procured from the market containing BB12 (*Bifidobacterium animalis subs lactis)* and La-5 (*lactobacillus acidophilus*) (Brand name: Mother Dairy Fruit & Vegetable Pvt Ltd., Mother Dairy, Patparganj, New Delhi).

The subjects screened for the study in group III will be asked to consume probiotic yogurt enriched with pumpkin seeds as a snack between breakfast and lunch.

Selected young adult females with PCOS will be categorized into four groups:

Group	**Intervention**	**Dietary exclusion**	**Intervention period**
Group I	200g of probiotic yogurt/day + Nutrition Counselling	Exclusion of any probiotics / conventional curd in the diet + pumpkin seeds	12 weeks
Group II	30 gm Pumpkin seed/day + Nutrition Counselling	Exclusion of any probiotics/ conventional curd in the diet + any nuts & seeds	12 weeks
Group III	Mix of 200 gm of probiotic yogurt+ 30 gm of pumpkin seeds/day + Nutrition Counselling	Exclusion of probiotics /conventional curd in the diet + any nuts & seeds	
Group IV (Control)	Nutrition Counselling	None	

The 12-week intervention has been selected as it has been observed in the study conducted by Arab et al. (2016) where probiotic supplementation for 12 weeks significantly reduced the Sex Hormone Binding Globulin

As mentioned above, after intervention once again anthropometric, biochemical, and dietary assessment will be done to analyze the changes achieved by the intervention. The results will be then interpreted.

Adherence to the intervention.

The World Health Organization (2003) has defined adherence as "the extent to which a person's behavior such as taking medication, following a diet, and executing lifestyle changes corresponds with agreed recommendations from a healthcare provider". Adherence percentage can act as an effective tool to measure the efficiency of the intervention provided.

Adherence to the intervention will be measured using a simple questionnaire and the responses will be recorded.

Adherence percentage = no of days followed the lifestyle advice and yogurt intake / total no of days * 100

Data Analysis

Both qualitative and quantitative data, incorporating descriptive and inferential methods for a comprehensive assessment of study outcomes will be analyzed by SPSS software (current version).

7. Expected Outcome of the Study

This research seeks to evaluate the effects of pumpkin seed-enriched probiotic yogurt, on inflammation biomarkers, insulin resistance, and hormonal indices among young women diagnosed with polycystic ovary syndrome. The study also seeks to assess the change in anthropometric measures as the secondary outcome. The study aims to provide insights into the potential benefits of this nutritional intervention in managing the complex interplay of factors associated with polycystic ovary syndrome, contributing to a better understanding of therapeutic approaches for this condition.

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